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Short Communication

Influenza antiviral resistance in the Asia-Pacific region during 2011

Sook-Kwan Leang [a], Yi-Mo Deng [a], Robert Shaw [a], Natalie Caldwell [a], Pina Iannello [a], Naomi Komadina [a], Philippe Buchy [b], Malinee Chittaganpitch [c], Dominic E. Dwyer [d], Peter Fagan [e], Ann-Claire Gourinat [f], Frances Hammill [g], Paul F. Horwood [h], Q.S. Huang [i], Peng Kei Ip [j], Lance Jennings [k], Alison Kesson [l], Tuckweng Kok [m], Jacob L. Kool [n], Avram Levy [o], Cui Lin [p], Katie Lindsay [q], Osmali Osman [r], Gina Papadakis [s], Fahimeh Rahnamal [t], William Rawlinson [u], Craig Redden [v], Jennifer Ridgway [w], I-Ching Sam [x], Suzanne Svobodova [y], Amado Tandoc [z], Geethani Wickramasinghe [aa], Jan Williamson [ab], Noelene Wilson [ac], Mohd Apandi Yusof [ad], Anne Kelso [a], Ian G. Barr [a], Aeron C. Hurt [a,ae,*]

[a] WHO Collaborating Centre for Reference and Research on Influenza, North Melbourne, Victoria, Australia
[b] Institut Pasteur, Phnom Penh, Cambodia
[c] National Institute of Health, Nonthaburi, Thailand
[d] Centre for Infectious Diseases and Microbiology Laboratory Services, ICPMR, Westmead Hospital, NSW, Australia
[e] Royal Darwin Hospital, Darwin, Australia
[f] Pasteur Institute, New Caledonia
[g] Queensland Health Forensic and Scientific Services, QLD, Australia
[h] Papua New Guinea Institute of Medical Research, Papua New Guinea
[i] WHO National Influenza Centre, Institute of Environmental Science and Research, Wellington, New Zealand
[j] Public Health Laboratory, Health Bureau, Macao SAR, China
[k] Canterbury Health, Christchurch, New Zealand
[l] The Children's Hospital at Westmead, Sydney, NSW, Australia
[m] Institute of Medical and Veterinary Science, South Australia, Australia
[n] World Health Organization, Division of Pacific Support, Suva, Fiji
[o] PathWest Laboratory Medicine, Nedlands, Western Australia, Australia
[p] National Public Health Laboratory, Ministry of Health, Singapore
[q] PathWest Laboratory Medicine WA, Princess Margaret Hospital for Children, Perth, Australia
[r] Virology Laboratory, Department of Laboratory Services, Ministry of Health, Brunei Darussalam
[s] Victorian Infectious Diseases Reference Laboratory, North Melbourne, Victoria, Australia
[t] Auckland District Health Board, Auckland, New Zealand
[u] Prince of Wales Hospital, Randwick, NSW, Australia
[v] Melbourne Pathology, Victoria, Australia
[w] The Canberra Hospital, ACT, Australia
[x] University Malaya, Kuala Lumpur, Malaysia
[y] Austin Hospital, Victoria, Australia
[z] Research Institute of Tropical Medicine, Manila, Philippines
[aa] NIC Sri Lanka, Medical Research Institute, Colombo, Sri Lanka
[ab] Royal Hobart Hospital, Tasmania, Australia
[ac] HAPS Pathology, Newcastle, NSW, Australia
[ad] Institute for Medical Research, Kuala Lumpur, Malaysia
[ae] Monash University, School of Applied Sciences, Churchill, Victoria, Australia

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ABSTRACT

Despite greater than 99% of influenza A viruses circulating in the Asia-Pacific region being resistant to the adamantane antiviral drugs in 2011, the large majority of influenza A (>97%) and B strains (~99%) remained susceptible to the neuraminidase inhibitors oseltamivir and zanamivir. However, compared to the first year of the 2009 pandemic, cases of oseltamivir-resistant A(H1N1)pdm09 viruses with the H275Y neuraminidase mutation increased in 2011, primarily due to an outbreak of oseltamivir-resistant viruses that occurred in Newcastle, as reported in Hurt et al. (2011c, 2012a), where the majority of the resistant viruses were from community patients not being treated with oseltamivir. A small number of influenza B viruses with reduced oseltamivir or zanamivir susceptibility were also detected. The

* Corresponding author at: WHO Collaborating Centre for Reference and Research on Influenza, 10 Wreckyn St, North Melbourne, Victoria 3051, Australia. Tel.: +61 3 9342 3914; fax: +61 3 9324 3939.
E-mail address: aeron.hurt@influenzacentre.org (A.C. Hurt).

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increased detection of neuraminidase inhibitor resistant strains circulating in the community and the detection of novel variants with reduced susceptibility are reminders that monitoring of influenza viruses is important to ensure that antiviral treatment guidelines remain appropriate.

1. Background

Influenza antivirals are important for the prophylaxis and treatment of influenza, particularly in severely ill or high-risk patients with underlying health conditions. There are two classes of influenza antivirals currently approved in many countries, the M2 ion channel inhibitors (adamantanes) and the neuraminidase inhibitors (NAIs). As a result of widespread adamantane resistance in influenza A viruses (Barr et al., 2007; Bright et al., 2005), the NAIs are the only class of drug currently recommended for the treatment of influenza (CDC, 2011). Oseltamivir (Tamiflu) has been the most commonly used and widely available NAI worldwide while a second NAI, zanamivir (Relenza), has been used far less. Since the introduction of NAIs to the market in 1999, zanamivir-resistant viruses have been rarely detected (Gubareva et al., 1998; Ison et al., 2006), while oseltamivir resistance is more common (Hurt et al., 2009a; Meijer et al., 2009). Here we summarize the antiviral susceptibility of influenza A(H1N1)pdm09, A(H3N2) and B virus isolates collected in the Asia-Pacific region during 2011 through the WHO Global Influenza Surveillance and Response System (GISRS). While we recognise that the lack of surveillance data from larger countries in the region such as China (excluding Macau, SAR China), Korea and Japan may be a limitation of this report, the number and diversity of viruses tested from the 13 Asia-Pacific countries in this study represents a broad sample from which to assess the antiviral sensitivity of influenza viruses in the region.

2. Adamantane resistance

Adamantane susceptibility was determined for 344 influenza A viruses by sequencing for substitutions in residues 26, 27, 30, 31 or 34 of the M2 gene that have previously been associated with adamantane resistance (Bright et al., 2005). All A(H1N1)pdm09 viruses (136/136) and all except one A(H3N2) (167/168) virus tested contained the S31N M2 substitution known to confer adamantane resistance. Substitutions in residues 26, 27, 30 or 34 were not detected. The single adamantane-sensitive A(H3N2) strain was isolated from a two year old male patient in the Philippines and did not contain any of the M2 gene mutations known to be associated with adamantane resistance. These data suggest that the vast majority of currently circulating seasonal influenza A viruses in the Asia-Pacific remain resistant to the adamantanes, supporting the current advice recommending against their use (CDC, 2011).

3. NAI susceptibility of influenza A and B viruses

NAI susceptibility was determined for 1957 influenza A and 1125 influenza B virus isolates from 13 countries and territories in the Asia-Pacific region (Table 1). Viruses were cultured in Madin–Darby Canine Kidney (MDCK) cells and tested in a fluorescence-based neuraminidase (NA) enzyme inhibition assay (Hurt et al., 2012b) to yield an IC_{50} value (concentration required to inhibit NA activity by 50%).

Briefly, viruses were incubated in the presence of a range of oseltamivir or zanamivir concentrations at room temperature for 45 min. 2-(4-Methylumbelliferyl)-a-D-*N*-acetylneuraminic acid (MUNANA; Sigma–Aldrich Corp. St. Louis, MO, USA) was added at a final concentration of 100 μM and was incubated for 60 min. The reaction was stopped by the addition of 100 μL stop solution (0.14 M NaOH in absolute ethanol). The plates were read using a Labsystems Fluoroskan II fluorimeter set at 355 nm (excitation wavelength) and 465 nm (emission wavelength) and the IC_{50} of each virus determined using Robosage curve fitting software (GlaxoSmithKline).

Based on their IC_{50}, viruses were categorised as showing either "normal inhibition", "reduced inhibition", or "highly reduced inhibition" according to criteria recently recommended by the WHO GISRS Expert Committee for Antiviral Resistance in Influenza (WHO, 2012). These criteria categorise influenza A viruses as showing "reduced inhibition" if an IC_{50} is 10- to 100-fold greater, and "highly reduced inhibition" if an IC_{50} is 100-fold or greater than the mean IC_{50} of viruses with normal inhibition from the same subtype. For influenza B viruses the criterion for "reduced inhibition" is an IC_{50} 5- to 50-fold greater, and "highly reduced inhibition" if the IC_{50} is 50-fold or greater than the mean of normal influenza B strains (WHO, 2012).

In addition to the isolates tested in the NA inhibition assay, 273 clinical samples containing A(H1N1)pdm09 viruses that were not able to be cultured were analysed in a pyrosequencing assay for the presence of the H275Y NA mutation (NA amino acid numbering used throughout this paper for N1, N2 and B sequences is based on counting amino residues from the first methionine residue) (Deng et al., 2011). This is the most commonly detected oseltamivir-resistance mutation in N1-containing viruses (Hurt et al., 2011b).

Ninety-nine percent (3043/3082) of influenza A and B viruses tested from the Asia-Pacific in 2011 displayed normal inhibition by both oseltamivir and zanamivir. The mean oseltamivir and zanamivir IC_{50} values of A(H1N1)pdm09 and A(H3N2) viruses with normal inhibition remained less than 1 nM, whilst the mean zanamivir and oseltamivir IC_{50} values of influenza B viruses with normal inhibition were 1.7 nM and 12.2 nM, respectively, 3-fold and 30-fold higher than that of influenza A viruses (Table 2). These mean IC_{50} values are similar to our previous data, confirming that the base-line susceptibility of circulating viruses has not changed significantly over the last several years (Hurt et al., 2004, 2011b).

Table 1
Influenza A and B virus isolates from 2011 analysed in the NA inhibition assay.

Country	Influenza virus type/subtype/lineage			
	A(H1N1)pdm09	A(H3N2)	B Victoria[a]	B Yamagata[a]
Australia	913	551	672	12
Brunei	23	–	–	–
Cambodia	43	18	104	–
Fiji	2	–	16	–
Macau (SAR, China)	13	11	9	6
Malaysia	6	13	–	1
New Caledonia	5	4	–	–
New Zealand	39	102	185	3
Papua New Guinea	5	1	–	1
Philippines	54	18	15	15
Singapore	43	48	31	10
Sri Lanka	2	12	13	2
Thailand	14	17	28	2
Total	1162	795	1073	52

[a] Influenza B viruses from the antigenically distinct HA lineages represented by the strains B/Victoria/2/87 and B/Yamagata/16/88.

Table 2
Number and frequency of virus isolates identified in 2011 by the NA inhibition assay with normal, reduced or highly reduced oseltamivir and zanamivir inhibition.

NAI	Classification	A(H1N1)pdm09 (*n* 1162)	H3N2 (*n* 795)	B Victoria (*n* 1073)	B Yamagata (*n* 52)
Oseltamivir	Normal inhibition	1135 (97.7%) [0.4 ± 0.3 nM][*]	795 (100%) [0.3 ± 0.2 nM][*]	1070 (99.7%) [12.1 ± 6.4 nM][*]	52 (100%) [12.2 ± 5.3 nM][*]
	Reduced inhibition[a]	0	0	3 (0.3%)	0
	Highly reduced inhibition[b]	27 (2.3%)[c]	0	0	0
Zanamivir	Normal inhibition	1141 (98.2%) [0.3 ± 0.2 nM][*]	794 (99.9%) [0.5 ± 0.2 nM][*]	1072 (99.9%) [1.7 ± 0.7 nM][*]	52 (100%) [1.9 ± 0.9 nM][*]
	Reduced inhibition[a]	7 (0.6%)[d]	1 (0.1%)[d]	1 (0.1%)	0
	Highly reduced inhibition[b]	14 (1.2%)[d]	0	0	0

[*] Mean IC_{50} ± SD (nM) of all influenza A or B isolates showing normal inhibition against oseltamivir or zanamivir, based on a single IC_{50} determination for each virus.
[a] Influenza A viruses: IC_{50} = 10- to 100-fold greater than the mean IC_{50} of influenza A viruses from same subtype with normal inhibition; influenza B viruses: IC_{50} = 5- to 50-fold greater than the mean IC_{50} of influenza B viruses from same lineage with normal inhibition.
[b] Influenza A viruses: $IC_{50} \geqslant$ 100-fold of the mean IC_{50} of influenza A viruses from same subtype with normal inhibition; influenza B viruses: $IC_{50} \geqslant$ 50-fold of the mean IC_{50} of influenza B viruses from same lineage with normal inhibition.
[C] Seventeen of the 27 isolates were from the Newcastle outbreak. The remaining 10 isolates (0.9%) were from other regions and were not associated with the Newcastle outbreak.
[d] These isolates contained either a Q136K or Q136R NA substitution. These mutations appeared only in MDCK cell culture isolates, but not in clinical specimens.

4. A(H1N1)pdm09 viruses – NAI susceptibility

Twenty-seven A(H1N1)pdm09 viruses (2.3%) had oseltamivir IC_{50} values in the highly reduced inhibition range (Table 2). All of these viruses contained a H275Y NA substitution, and had a mean oseltamivir IC_{50} value of 345 nM, 930-fold higher than that of viruses with normal inhibition, but remained susceptible to zanamivir. A further 15 A(H1N1)pdm09 H275Y variant viruses were detected in clinical specimens analysed by pyrosequencing, resulting in a total of 42 H275Y viruses detected from 1435 A(H1N1)pdm09 isolates or specimens tested (2.9%). Thirty-two of the H275Y variants were part of a cluster of cases centred around the city of Newcastle, NSW, Australia, which has been reported previously (Hurt et al., 2011c, 2012a).

Based on genetic analysis, the remaining 10 (0.9%) H275Y variants of 2011 were from Brunei (n = 1), the Philippines (n = 2) and various regions of Australia (n = 7) and were not considered part of the 'Newcastle cluster'.

Of the 42 H275Y 2011 cases, information regarding antiviral treatment was available for 36 patients. The majority of these patients (33/36, 91%) were from the community and were not being treated with oseltamivir, whereas in 2009–2010 the majority of H275Y cases (with known treatment information) (15/23, 65%) were from hospitalised patients undergoing oseltamivir treatment (Hurt et al., 2011b) (Fig. 1). This increase in H275Y virus detection in patients with no known oseltamivir exposure has also been seen in the United States and United Kingdom during their 2010–2011 influenza seasons (summarised in Fig. 1) (Lackenby et al., 2011; Storms et al., 2012), and may suggest a change in the ability of H275Y A(H1N1)pdm09 viruses to transmit in the absence of oseltamivir selective pressure.

Although 21/1141 (1.8%) A(H1N1)pdm09 tested had reduced or highly reduced zanamivir inhibition (Table 2), all of these 21 isolates contained either a Q136K or Q136R NA substitution that appeared to arise only during MDCK cell culture, as analysis of the corresponding clinical specimen failed to detect these substitutions. If this is taken into account, none of the 2011 A(H1N1)pdm09 viruses found in patient samples would be expected to have reduced zanamivir inhibition. The Q136K variant has been shown previously to arise in seasonal A(H1N1) viruses during MDCK cell culture (Hurt et al., 2009b; Okomo-Adhiambo et al., 2010), and therefore the detection of Q136K and Q136R

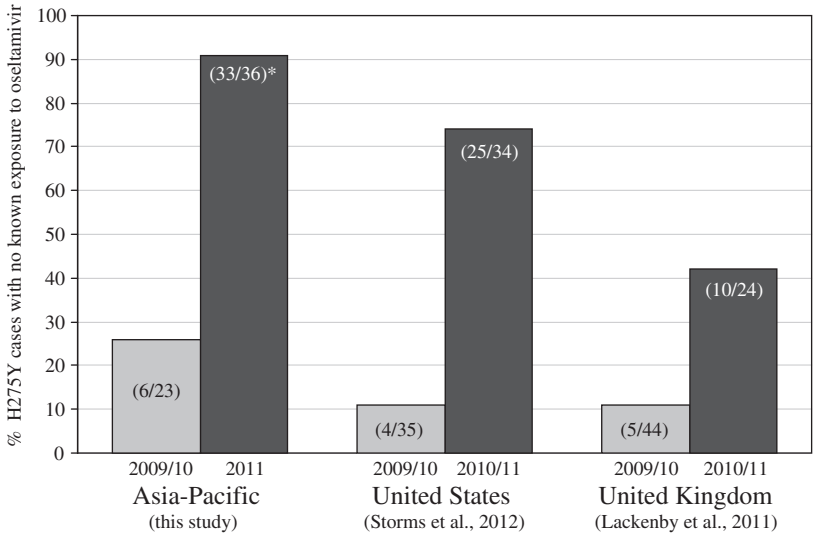

Fig. 1. Oseltamivir-resistant A(H1N1)pdm09 H275Y cases with no known exposure to oseltamivir. Since the first year of the 2009 pandemic the number of oseltamivir-resistant A(H1N1)pdm09 H275Y cases with no known exposure to oseltamivir or contact with known cases of oseltamivir resistance has increased in the Asia-Pacific (this study and Hurt et al., 2011b), the United States (Storms et al., 2012) and the United Kingdom (Lackenby et al., 2011). *31 of the 33 H275Y untreated cases were related to the Newcastle outbreak.

Table 3
Influenza A(H3N2) and B viruses with reduced or highly reduced inhibition in 2011.

Designation	Subtype/type/lineage	NA mutation(s)	Inhibition category	Oseltamivir IC_{50} (nM)* (fold difference)[a]	Zanamivir IC_{50} (nM)* (fold difference)[a]
A/Victoria/649/2011	A(H3N2)	Q136K[c]	Reduced inhibition	0.1 ± 0.0	13.5 ± 0.8 (27)
B/Philippines/2519/2011	B Victoria	A245S[b]	Reduced inhibition	83.3 ± 7.2 (7)	0.6 ± 0.1
B/Wellington/39/2011	B Victoria	I221T[b]	Reduced inhibition	221.6 ± 46.6 (18)	2.1 ± 0.0
B/Waikato/21/2011	B Victoria	A245T[d]	Reduced inhibition	237.9 ± 31.8 (20)	54.5 ± 9.7 (32)

* Mean IC_{50} ± SD (nM), each virus tested in triplicate.
[a] Fold increase relative to the mean IC_{50} of viruses of that type/subtype and only included when $\geqslant 5$.
[b] Detected in clinical specimen and virus isolate.
[c] Only detected in virus isolate.
[d] Clinical specimen not available.

substitutions in A(H1N1)pdm09 viruses serves as a reminder to check the sequence of viruses in clinical specimens after an isolate has been identified with an increased IC_{50}.

In early 2011, we also detected a small cluster of viruses ($n = 22$) isolated from cases in northern Australia and Singapore that contained a S247N NA substitution (Hurt et al., 2011a). Although the S247N mutation alone conferred only a mild increase in oseltamivir IC_{50} that did not reach the threshold for 'reduced inhibition' (mean oseltamivir IC_{50} value of S247N variants was 2.5 nM, 6-fold greater than viruses with normal inhibition), when combined with the H275Y mutation, the two mutations conferred an oseltamivir IC_{50} that was 8-fold higher than a virus with the H275Y mutation alone (Hurt et al., 2011a).

5. A(H3N2) viruses – NAI susceptibility

All 795 A(H3N2) isolates had normal oseltamivir inhibition and all except one had normal zanamivir inhibition. The one virus that showed reduced zanamivir inhibition contained a Q136K substitution that was present only in the MDCK isolate not in the clinical specimen, a similar finding to that seen in the A(H1N1)pdm09 isolates with this mutation. Taking this into account it would be expected that 0% of 2011 A(H3N2) viruses found in patients would be expected to have reduced inhibition to zanamivir.

6. B viruses – NAI susceptibility

Of the 1125 influenza B viruses tested, three showed reduced inhibition, all of which were from the B/Victoria/2/87 HA lineage (Table 3). Two viruses with reduced oseltamivir inhibition, one from the Philippines and the other from New Zealand, had an oseltamivir IC_{50} of 83 nM and 222 nM, respectively, 7- to 18-fold higher than B viruses with normal inhibition. The Philippines virus contained a A245S NA mutation, while the New Zealand strain contained an I221T NA mutation, a substitution detected recently in a number of influenza B viruses from North Carolina during the 2010/11 USA influenza season (Sleeman et al., 2011). The only influenza B virus isolate that showed reduced zanamivir inhibition was B/Waikato/21/2011, which had a 20-fold reduction in oseltamivir susceptibility and a 32-fold reduction in zanamivir susceptibility, and contained an A245T NA mutation at the same residue as the B/Philippines/2519/2011 virus which had an A245S mutation and normal zanamivir inhibition (Table 3). While the roles of these novel influenza B mutations in NAI susceptibility need to be confirmed by reverse genetics experiments, it is interesting to note that the NA residue A245 in influenza B is equivalent to residue S247 in the A(H1N1)pdm09 viruses (Nguyen et al., 2012), the site of the S247N mutation that conferred a minor reduction in susceptibility in Australian and Singaporean A(H1N1)pdm09 viruses as described previously (Hurt et al., 2011a).

7. Conclusions

Surveillance for antiviral susceptibility of influenza viruses from 2011 in the Asia-Pacific showed that NAI resistance in A(H1N1)pdm09, A(H3N2) and B viruses remained low, although >99% of influenza A strains continued to be resistant to the adamantane drugs. The sporadic episodes of community transmission of viruses with reduced oseltamivir susceptibility such as S247N or, more importantly, H275Y variants raise concerns that these strains may spread globally. The Newcastle cluster of H275Y cases represented the most widespread emergence of A(H1N1)pdm09 oseltamivir-resistant viruses in a community setting to date and contributed significantly to the number of H275Y cases detected in this study. Without the Newcastle outbreak, no increase in community cases of H275Y would have been detected in the Asia Pacific region in 2011. The frequency A(H3N2) and influenza B viruses showing reduced NAI inhibition continues to be much lower than that seen for A(H1N1)pdm09 viruses, although a small number of novel B variants were identified which warrant further analysis. Because wild type influenza B viruses have an oseltamivir IC_{50} that is already 30-fold higher than that of influenza A viruses, B viruses should be monitored for NA mutations that shift the IC_{50} even higher, potentially causing a loss in drug effectiveness. By focusing surveillance efforts on community specimens, the chances of rapidly detecting clusters of resistant viruses that may be spreading will be improved. Continued surveillance remains important throughout the Asia-Pacific region to ensure the appropriate use of influenza antiviral drugs.

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